

IDENTIFICATION AND PHYLOGENETIC ANALYSIS OF ENTOMOPATHOGENIC FUNGAL ISOLATES USING MOLECULAR APPROACH

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Abstract: Entomopathogenic fungi are essential to consider as one of the biological agents to control termite populations. This research aimed to molecularly identify entomopathogenic fungi isolates in termites based on the ITS rDNA region and to determine the relationship of fungi isolates. Identification was performed by DNA extraction, PCR amplification, electrophoresis, purification, and sequencing. Phylogenetic trees were generated using MEGA X. Molecular identification showed that the ISO1 sample was *Penicillium oxalicum*, the ISO2 sample was *Trichoderma ghanense* the ISO3 sample was *Aspergillus niger*, the ISO4 sample was *Aspergillus fumigatus* and the ISO5 sample was *Aspergillus pseudonomius*. The phylogenetic tree showed that the ISO1, ISO2, ISO3, ISO4, and ISO5 samples had the closest relationship with *Penicillium oxalicum* strain FR6-CGR12, *Trichoderma ghanense* isolate TM2, *Aspergillus niger* isolate 77, *Aspergillus fumigatus*, and *Aspergillus pseudonomius* strain DTO 267D6, respectively.

Keywords: Entomopathogen; molecular identification; phylogenetic tree

Abstrak: Kelimpahan jenis fungi entomopatogen adalah hal yang terpenting untuk dipertimbangkan sebagai agen hayati bagi populasi rayap. Penelitian ini bertujuan untuk mengidentifikasi secara molekular isolat fungi entomopatogen pada rayap, berdasarkan daerah ITS rDNA dan mengetahui hubungan kekerabatan dari isolat fungi tersebut. Identifikasi dilakukan dengan cara ekstraksi DNA, amplifikasi menggunakan PCR, elektroforesis, purifikasi dan sekuensing. Selanjutnya kontruksi pohon filogenetik menggunakan aplikasi MEGA X. Berdasarkan uji molekular menunjukkan bahwa sampel ISO1 merupakan *Penicillium oxalicum*. ISO2 merupakan *Trichoderma ghanense*. ISO3 merupakan *Aspergillus niger*. ISO4 merupakan *Aspergillus fumigatus*. ISO5 merupakan *Aspergillus pseudonomius*. Konstruksi pohon filogenetik menunjukkan bahwa, sampel ISO1 berkerabat dekat dengan *Penicillium oxalicum* strain FR6-CGR12. Sampel ISO2 berkerabat dekat dengan *Trichoderma ghanense* isolat TM2. Sampel ISO3 berkerabat dekat dengan *Aspergillus niger* isolat 77. Sampel ISO4 berkerabat dekat dengan *Aspergillus fumigatus*. Sampel ISO5 berkerabat dekat dengan *Aspergillus pseudonomius* strain DTO 267D6.

Kata kunci: Entomopatogen; identifikasi molekular; pohon filogenetik

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Introduction

The abundance of entomopathogenic fungi is the most important thing to consider as a biological agent for termite populations. Entomopathogenic fungi groups that have been widely reported include Basidiomycota, Ascomycota, Entomophthoromycota, Chytridiomycota, and Microsporidia. Ascomycota groups are the most common types of fungi, which are 180 entomopathogens that are known to infect insects such as *Metarhizium anisoplae*, *Trichoderma* sp., *Penicillium* sp., and *Aspergillus* (Gherbawy and Voigt, 2010; Sintawati *et al.*, 2016; Trizelia *et al.*, 2015).

A survey conducted at Alur Mancang, Seulawah Ecosystem, found one isolate that was potential as an entomopathogen; namely *Aspergillus flavus* (Lisa, 2019). Moreover, Hanum (2020) found nine isolates that were potentially entomopathogenic to control termites from the Suaq Balimbing area, Gunung Leuser National Park, Aceh Province.

The macroscopic and microscopic identification of these isolates faced many obstacles, especially the influence of various environmental factors that often lead to morphological characters. In addition, these identification techniques such as this cannot describe genetic relationships between types of individuals genetically (Gherbawy and Voigt, 2010), thus, molecular identification is needed to support the accuracy of macroscopic and microscopic identification. In addition, molecular identification can distinguish genetic variations or DNA polymorphisms of an individual (Rajaratnam and Thiagrajan, 2012), and will show more accurate and efficient results. The use of DNA in phylogenetic analysis has several advantages including more accurate data, providing many character states due to substantial base changes, and producing a more natural kinship relationship (Hennig, 2016).

Polymerase Chain Reaction (PCR) is a technique commonly used in molecular identification. The PCR technique amplifies target DNA in vitro with the help of the DNA polymerase enzyme, synthesizing of new DNA from 5' to 3' via a single strand. Also, this PCR technique can produce high specificity and a fast process (Joachim *et al.*, 2006).

This study aimed to molecularly identify entomopathogenic fungi isolates associated with termites (ISO1, ISO2, ISO3, ISO4, and ISO5), based on the ITS rDNA region and determine the kinship relationship of the fungi isolates.

Materials and Methods

Collection of Samples

The entomopathogenic fungi isolates were collected from Microbiology Laboratory, Syiah Kuala University, Aceh Province. The entomopathogenic fungal isolates were recovered from a previous culture, then grown in the PDA media.

DNA Extraction and PCR Amplification

The DNA extraction from the fungi entomopathogenic was performed using Quick-DNATM Fungal / Bacterial Miniprep Kit (Zymo Research, USA. Catalog No. D6005). The DNA quality and quantity tests were measured using Nanodrop 2000C. Primers (ITS1 and ITS4) were used to amplify ribosomal internal transcribed spacer (ITS).

Sequencing and Phylogenetic Analysis

The PCR product was sent for sequencing to PT. Genetika Science. The obtained sequences were compared with the other related sequences using BLAST search in GenBank (NCBI). The BLAST output was processed for generating the phylogenetic tree in software MEGAX by using 'Neighbor Joining'.

Result and Discussion

Isolation of Entomopathogenic Fungi DNA Associated with Termites

The results of the isolation of entomopathogenic fungi DNA associated with termites showed that DNA ISO2, ISO3, ISO4, and ISO5 had good concentration levels of 100-250 ng / μ l and also good purity of 1.8-1.9. This differs from ISO1 which has a low DNA concentration level of 59.1 ng / μ l, but a good purity level of 1.92 (Table 1). The small amount of DNA concentration produced by ISO1 might be due to errors in processing such as the addition of solution and the speed of the extraction process. Thermo Scientific (2016), the absorbance value of DNA is measured at a wavelength of 260 nm, with a good amount of DNA concentration to be used in the amplification process that is 100-500 ng/ μ l. DNA with a good level of purity ranges from 1.8 to 2.2. Purity values <1.8 or> 2.0 indicate that the DNA is still not pure or contaminated.

Table 1. DNA Concentration and Purity Value of Fungi Isolates Entomopathogens Associated with Termites

Samples	Concentration Value (ng/ μ l)	Purity Value (A260/280)
ISO1	59.1	1.92
ISO2	210.2	1.91
ISO3	141.6	1.88
ISO4	235.9	1.89
ISO5	135.4	1.88

Koetsier *et al.*, (2019), The absorbance ratio at A260 / 280 wavelength (<1.85-1.88) indicates the presence of proteins or other organic compounds contamination, but if the ratio value (> 2.1) indicates the presence of RNA contamination. Errors in addition to the solution during DNA isolation might significantly affect the purity and concentration of DNA so that it will give poor results during the DNA amplification process.

Amplification of ITS Region Using PCR Technique

The DNA amplification showed that DNA band sizes at 400 - 600 bp (Figure 1). The observations showed that the five samples (ISO1, ISO2, ISO3, ISO4, and ISO5) produced different band thicknesses and smears. This is because the concentration and purity values in each sample are different (Table 1). However, the ISO1 sample produced a thinner DNA band when compared to the other samples (Figure 1). Based on the DNA concentration test results, ISO1 obtained a DNA concentration value of 59 ng/ μ l, it shows that the value of DNA concentration was still below the supposed value of 100-500 ng/ μ l. Based on this, it shows that the concentration value that slightly affects the thickness of the DNA band after the amplification process.

Figure 1 shows that the five samples (ISO1, ISO2, ISO3, ISO4, and ISO5) still have a smear indicating the presence of other compounds that are carried during the extraction process, or DNA that was degraded. Magdeldin (2012) and Lee *et al.* (2012), states that excessive movement during processing, certain chemical compounds, and suboptimal temperature during the extraction process can cause DNA molecules to degrade into smaller parts, thus causing DNA damage. Besides, the materials used during the extraction process, protein or RNA might be also the cause of the smear formation.

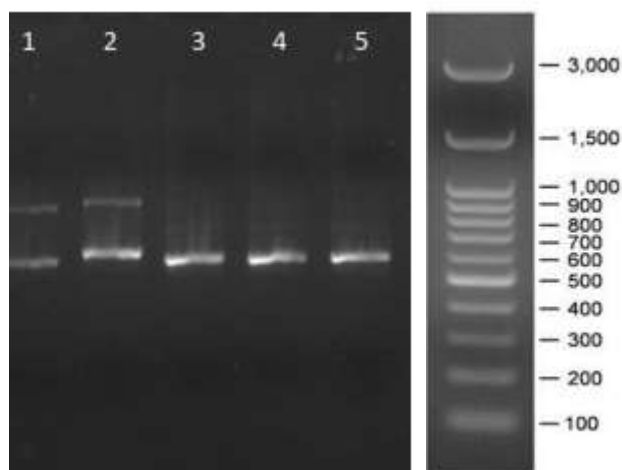


Figure 1. Results of ITS rDNA Gene Amplification (M) Markers (1) ISO1 Samples (2) ISO2 Samples (3) ISO3 Samples (4) Samples ISO4 (5) ISO5 Samples

ITS Sequences Analysis

Table 2. Identification of Fungal Isolates of ITS Region

Samples	Species Identified	Identity
ISO1	<i>Penicillium oxalicum</i>	99.29%
ISO2	<i>Trichoderma ghanense</i>	99.05%
ISO3	<i>Aspergillus niger</i>	99.59%
ISO4	<i>Aspergillus fumigatus</i>	99.60%
ISO5	<i>Aspergillus pseudonomius</i>	99.16%

The molecular identification was carried out by DNA barcoding using the ITS region sequencing. The ITS rDNA sequences were compared to those in the databases using NCBI-BLAST. Five species were identified using DNA barcoding with an identity range between 99.05 – 99.60%. The identification of fungi that have been carried out macroscopically and microscopically by Hanum (2017) and Lisa (2017) produces the same data as molecular identification carried out in this study, where the ISO1 sample is *Penicillium* sp., ISO2 is *Trichoderma* sp., ISO3 is *Aspergillus niger*, while ISO4 is *Aspergillus fumigatus*.

Macroscopic and microscopic testing of ISO5 samples showed the type of *Aspergillus flavus*, but in the molecular test, it showed type *A. pseudonomius*. However, when viewed from the BLAST results, *A. flavus* had a Percentage Identity value of 98.95%. This shows that the ISO5 sample still bears similarity to the type of *A. flavus*. However, the molecular identification results in the ISO5 sample show that the type of *A. pseudonomius* with a higher Percentage Identity value is 99.16%. According to Stackebrandt and Goebel (1994), the higher the value of Percentage Identity indicates the higher similarity of DNA sequences. Phylogenetic tree constructed from the BLAST result of NCBI showed the fungi samples (ISO1, ISO2, ISO3, ISO4, ISO5) were closer to the sequence in the NCBI database.

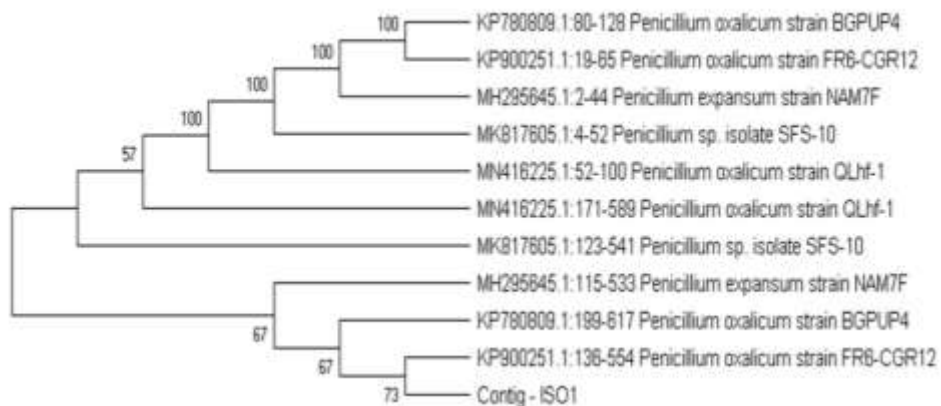


Figure 2. Molecular phylogenetic analysis of ISO1 sample

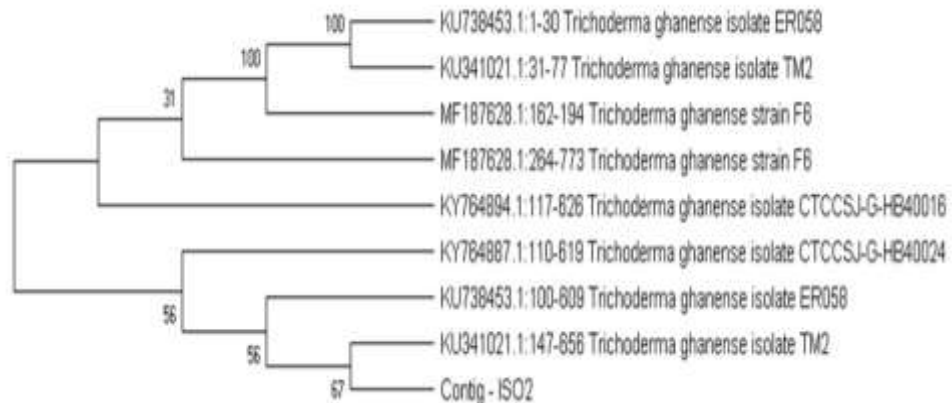


Figure 3. Molecular phylogenetic analysis of ISO2 sample

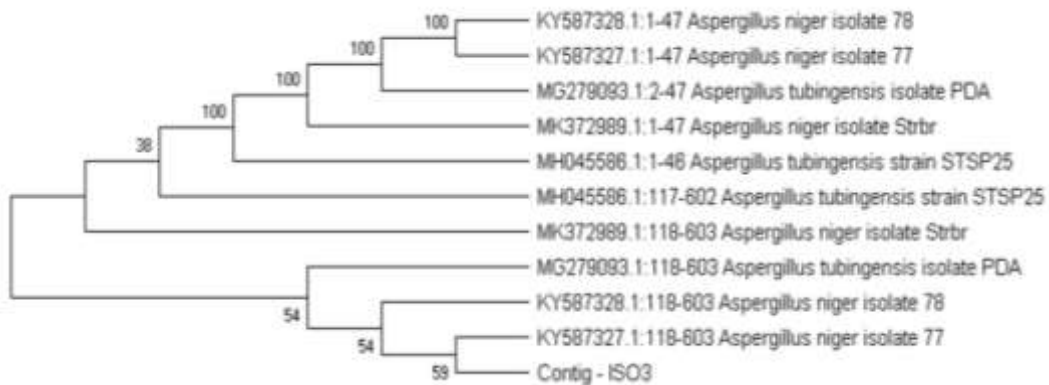


Figure 4. Molecular phylogenetic analysis of ISO3 sample

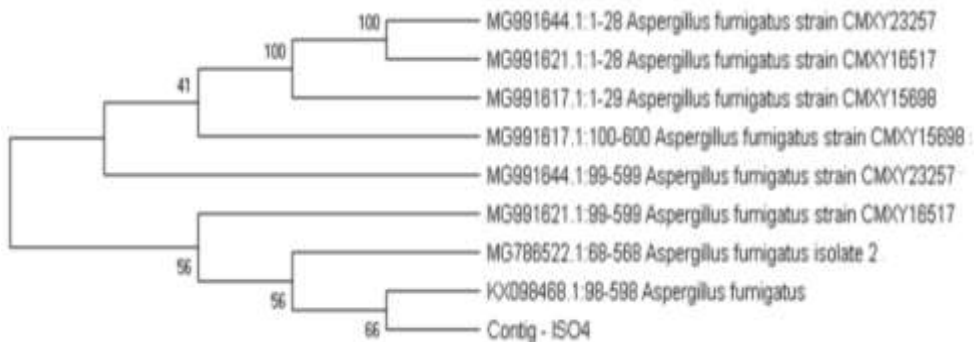


Figure 5. Molecular phylogenetic analysis of ISO4 sample



Figure 6. Molecular phylogenetic analysis of ISO5 sample

Apollos et al. (2017) and Huang et al. (2009) states that the bootstrap value between 70-100 shows the branching formed is sturdy and difficult to change again. Yet, if the bootstrap value is less than 70, then the possibility of changes in the branching formed is very high. Russo and Selvatti (2018) signified that, a bootstrap value <50 indicates a very weak nature of the branching formed. According to Reddy (2011), the same branching shows a monophyletic group, meaning that it comes from one common ancestor. Generally, monophyletic groups have the same genetic and biochemical properties, so they have a very close kinship.

Conclusion

Phylogenetic analysis of the five samples of entomopathogenic fungi isolates collected from termite nests. The ISO1, ISO2, ISO3, ISO4, and ISO5 samples were *P. oxalicum*; *T. ghanense*; *A. niger*; *A. fumigatus*, and *A. pseudonomius* respectively. Phylogenetic tree construction shows that the five isolate samples (ISO1, ISO2, ISO3, ISO4, and ISO5) have a closed relationship with the sequence in the NCBI database.

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